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OBTAINING BIO-ACTIVE EXTRACELLULAR POLYSACCHARIDE FROM *CORDYCEPS SINENSIS*

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SUMMARY OF DOCTORAL DISSERTATION OF BIOTECHNOLOGY

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PREFACE

1. Rationale of the study

Currently, there are many pharmaceutical products and functional foods developed from *Cordyceps sinensis*. Although *C. sinensis* biomass production technology brings great economic value, another source of benefits from culture fluid seems to be forgotten. Obviously, the disposal of culture fluid not only increases the cost of environmental treatment, but also loses a valuable source of biologically active substances - exopolysaccharide (EPS). Many studies have shown that EPS possesses many biological activities such as immunomodulatory, anti-tumor, anti-oxidative and help hypoglycemic. Researchs of obtaining EPS from the culture of *C. sinensis* haven't had much. Information about biologically active ingredients, including EPS, is still limited. Moreover, the active substances produced by *C. sinensis* depend a lot on the environment, conditions and culture method. In present, information about the EPS active ingredients being secreted by the fungus *C. sinensis* in the liquid-static culture medium is still very limited, especially in Vietnam. Therefore, comprehensive studies of the basic liquid-static culture medium of C. sinensis combined with the addition of fungal biomass stimulating factors and EPS are urgently needed. The biological activity of EPS in general and the EPS segments in particular depends on their monomeric structure, chemical structure and size. Currently, the information is still very limited, especially information about the frame structure as well as the branch circuit of the EPS segments. That why it is necessary to conduct investigations related to the acquisition, purification, assessment of biological activity and the skeleton structure of EPS segments obtained from the liquid-static culture of *C. sinensis* .

Because of the above reasons, this dissertation focuses on researching the liquid-static culture of *C. sinensis* as a basis for optimizing the technological process of producing this medicinal fungi in Vietnam. Research on obtaining EPS and bioactive EPS segments from fungal culture fluid and studying their bioactivity enhancement.

2. Aim of the dissertation's research

Development of a process for obtaining the extracellular polysaccharide of *Cordyceps sinensis* with biological activity

3. The main research contents of this dissertation

- Contents 1: Survey of appropriate nitrogen sources in C. sinensis culture to obtain bioactive EPS and culture to increase EPS biosyndissertation by addition of vegetable oil.
- Contents 2: Survey of acquisition, purification and modification sulfate to enhance the bioactivity of EPS segments.
- Contents 3: Survey of the composition and chemical structure of EPS obtained from *C. sinenesis* stationary liquid culture.

- Contents 4: Research on proposing a technological process for chemically cultured liquid and static *C. sinensis* to produce biologically active EPS.

CHAPTER 1 . OVERVIEW

1.1. Overview of exopolysaccharide

EPS obtained from *C. sinensis* cultures significantly reduced the expression of c-Myc, c-Fos and vascular membrane development factor in mice with melanine B16. It shown that EPS could stop tumors from growing in the lungs and liver of mice and could be a potential solution in cancer therapy. EPS exhibits antitumor activity primarily through the enhancement and activation of the host's immune response. An EPS extracted from an asexual C. sinensis strain of Tolypocladium sinensis may inhibit the metastasis of melanine B16 cells in the lungs and liver. In addition, this EPS was able to suppress H22 tumor growth and increase immune activity in mice carrying H22 tumors, suggesting that EPS suppresses tumors by activating the body's immune system.

1.2. Improve the bioactivity of polysaccharide

There are many methods of sulfating polysaccharides such as oleum-dimethylformamide (DMF) method, SO3-pyridine method, chlorosulfonic acid (CSA)-pyridine (Pyr) method and aminosulfonic acid (ASA)-pyridine (Pyr) method [23]. Among them, CSA-Pyr method is often applied: The mixture of dissolved polysaccharide and CSA-Pyr complex is reacted under suitable temperature conditions for a certain period of time with a controlled CSA/Pyr ratio. For poorly soluble polysaccharides, they should be dispersed in ionic solution before reacting with CSA-Pyr complex. The disadvantage of this method is that CSA has strong oxidizing properties and long reaction time can break down the structure of polysaccharide [56]. Recently, some derivatives of polysaccharide sulfate from medicinal fungi/plants have been synthesized by CSA/Pyr method. The research results show that the biological activity of sulfate derivatives is much higher than natural polysaccharide. With *C. sinensis* object, Yan et al. obtained S-EPS-1D derivatives by CSA/Pyr method, which has higher ABTS+ and hydroxyl free radical binding activity than natural EPS [61].

1.3. Identify segments and analyze the resulting EPS component

Hwang et al. (2003), EPS obtained after precipitation with ethanol was dissolved in 0.2M NaCl to create EPS solution with a concentration of 10 g/l, and loaded in a Sepharose CL–4B column (2, 4 x 100cm; Sigma Chemical Co., St Louis, MO, USA). Su et al (2013), the EPS sample obtained after precipitation with ethanol was loaded in a Sephacryl S-300 HR column (2.6 x 100 cm, Pharmacia Co.) and lysed with 10 mM NaCl solution at a speed flow of 1 ml/min. The EPS segments were collected and freeze-dried, kept at -20 $^{\circ}$C for further study. Hydrolyzate about 2 mg of the polysaccharide obtained with trifluoroacetic acid (TFA) for 10 hours at 110 ^{0}C. The excess acid is removed with methanol, and the hydrolysate is analyzed with the HPLC/ELSD system. The results were compared with standard monosaccharides: D–glucose, L–rhamnose, D–xylose, D–mannose and L–arabinose.

1.4. Determination bonds, structure of exopolysaccharide

The purified EPSs were used for further structural and activity studies. Now, to better understand EPS, researchers have performed structural analysis of this molecule. The primary structure of the carbohydrate complex has been elucidated in studies of: 1) monosaccharide composition, 2) structure of linkages, 3) ring size, 4) stereochemistry, 5) sequence mono-radical and repeating units, 6) metabolic groups, 7) molecular size distribution.

From the research objective of the dissertation presented above, the dissertation proposes a research process consisting of 4 contents modeled in the general research diagram. This research diagram will serve as a theoretical and empirical basis for proposing a technological process for liquid and static culture of C. sinensis fungus in an improved medium with the addition of vegetable oils to produce high biomass and EPS yields. EPS complex acquisition study and EPS segments. Study of simple sugar composition, chemical structure and modification of bioactivity of EPS segments.

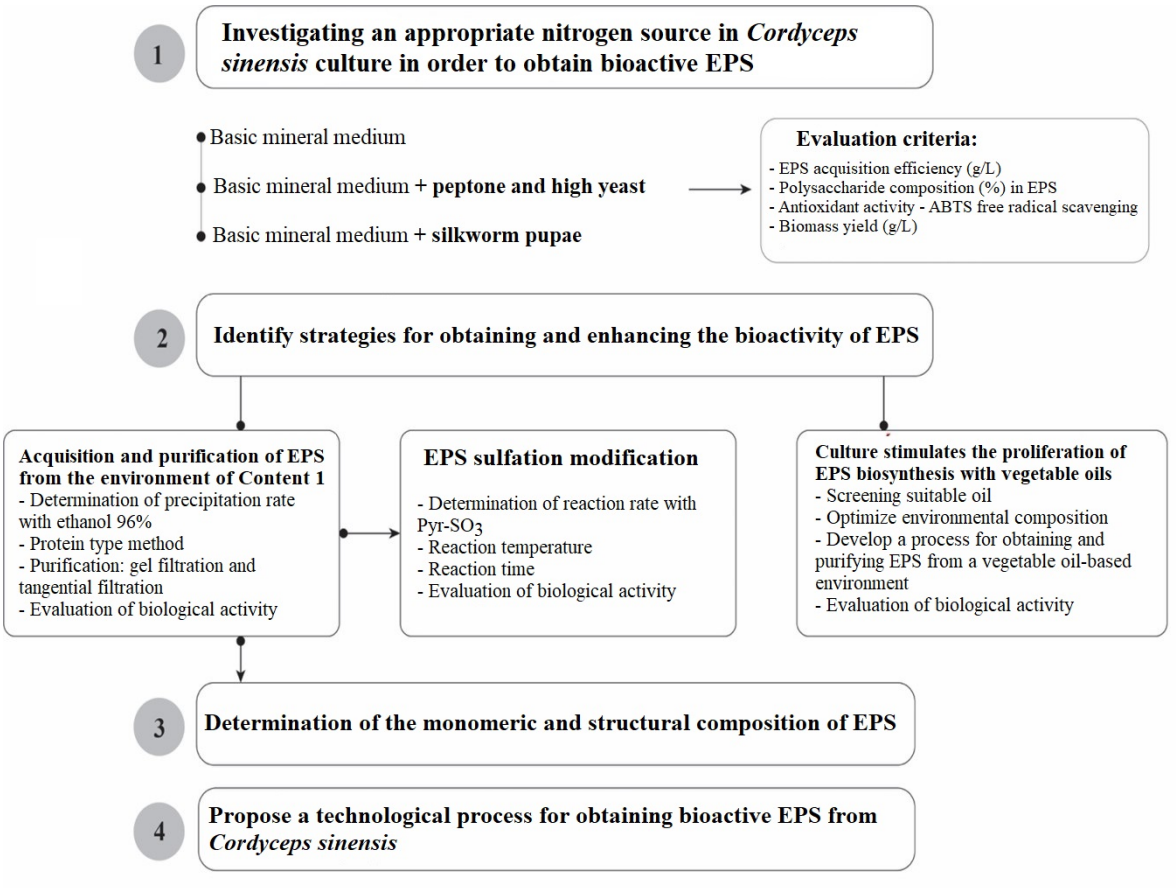

Figure 2.1. General diagram of the dissertation research

CHAPTER 2 . MATERIALS AND METHODS

2.1. Research Materials

Cordyceps sinensis CS-YK2007 was provided by Dr. Truong Binh Nguyen, Institute of High-tech Agricultural Research and Application, University of Da Lat, Lam Dong. The fungus *C. sinensis* was cultured at the Biochemistry laboratory of the University of Natural Sciences in Ho Chi Minh City. In the process of carrying out the research, using liquid-static culture fluid prepared by Dr. Provided by Truong Binh Nguyen.

2.2. Research Methods

2.2.1. Investigate suitable nitrogen sources in C. sinensis fungal culture to obtain bioactive EPS

C. sinensis culture process typically last 30-40 days in different medium: MT1 (basic potato and saccharose), MT2 (MT1 + peptone and yeast high) and MT3 (MT1 + pupae). Monitor the development of each experiment by recording biomass morphology, commenting on growth rate, biomass collection time and EPS after 10, 20, 30, 40 days.

2.2.2. Static liquid culture method in medium supplemented with olive oil

Medium preparations: potato (200 g/l), sucrose (50 g/l), yeast extract (4 g/l), peptone (6 g/l), KH 2 PO 4 (0.5 $_{g}$ / $_{l}$) l), K $_{2}$ HPO $_{4}$ (0.5 g/l), CaCl $_{2}$ (0.5 g/l), MgSO $_{4}$ (0.2 g/l). pH 6-7. Mix olive oil and Tween 80: ratio 1 ml: 0.3 ml . Culture at 20 - 25 ^{o} C, for 40 days. The collected solution was autoclaved to collect EPS.

2.2.3. Extraction of EPS from Cordyceps sinensis culture fluid supplemented with oil

2.2.3.1. Extraction of exopolysaccharide from Cordyceps sinensis culture fluid supplemented with olive oil

Exopolysaccharide (EPS) extraction process from *Cordyceps sinensis* culture based on the method of Kim and Yun (2003); Sharma Sapan Kumar et al. (2015) have modifications related to the addition and removal of oil from the culture.

2.2.3.2. Investigation of organic solvents to remove oil from the solution by liquid-liquid extraction technique

Survey of 3 organic solvents: petroleum ether, hexane, diethyl ether. Add 3 solvents of petroleum ether, hexane, diethyl ether to each jar at a ratio of 1:1. Shake the pitchers well for a homogeneous sample and solvent solution. For peaceful slang, observe the separation of layers. Collect the translation below after 40 minutes. Repeat the process 4 times. Determination of polysaccharide and lipid content before and after oil removal. Determination of ABTS+ free radical arresting activity of EPS sample before and after oil removal.

2.2.3.3. Investigation of ethanol precipitation rate 96 ^{0} to obtain exopolysaccharide from lip school add oil _

The culture fluid after the oil was precipitated with ethanol 96 ^{0} in the ratio 1:3; 1:4; 1:5. Determine polysaccharide and protein content in each experimental batch.

2.2.3.4. Protein removal method

❖ **Protein removal by sevag . method**

Protein extraction was carried out according to the method of Huang et al (2010) with several steps adjusted to suit laboratory conditions [14]. Reagent sevag (Chloroform: Buthanol) with three survey ratio 3:1 ; 4:1 and 5:1

Determine protein and polysaccharide content before and after protein removal with sevag. Determine ABTS $^{+}$ free radical scavenging capacity of EPS samples before and after protein removal

❖ **Using TCA to remove proteins from EPS samples**

The protein removal procedure was carried out according to the method of Huang et al (2010) with several steps adjusted to suit the laboratory conditions. Dissolve raw EPS with water, incubate with TCA 10%, 20%, 30%, 40% adjust the mixture to pH 3.0

Determine protein and polysaccharide content before and after protein removal. Determination of ABTS $^{+}$ free radical scavenging capacity of EPS samples before and after protein removal.

❖ **Using protease enzyme to remove protein from EPS sample**

Dissolve the raw EPS sample in water, mix with Alcalase enzyme 10U, 20U, 30U, 40U, 50U in the ratio 3:1 v/v. Determine protein and polysaccharide content before and after protein removal. Determine ABTS $^{+}$ free radical scavenging capacity of EPS samples before and after protein removal.

2.2.4. EPS segment acquisition

2.2.4.1. Obtaining EPS segment by Tangential Flow Filtration method

Put float fluid through a Tangential Flow Filtration system, the membrane has NMWC pore sizes of 750 kDa and 30 kDa; and membranes with 100 kDa and 750 kDa NMWC pore sizes.

2.2.4.2. Obtaining the EPS segment by gel filtration chromatography

Load the EPS sample (10 g/l) into a chromatographic gel column with h=70cm, d=2.4cm, Sephadex-G100 gel. Determine the polysaccharide and protein content of each tube.

2.2.5. Modification of sulphation EPS

2.2.5.1. Create guide produce sulphated EPS

Perform the sulfation reaction according to the following parameters: The three parameters include the EPS/SO3 ratio. Py (1:5, 1:7, w/w), time (1.5h, 2h)

2.2.5.2. Method for determination of substitution (DS)

Based on the sulfur content, the DS substitution is calculated by the following formula:

$$DS = \frac{1{,}62 \times S\%}{32 - 1{,}02 \times S\%}$$

In which, S% is the sulfur component of sulfate derivatives.

The acquisition yield of sulfur derivatives is calculated by the following formula:

$$HS\% = \frac{Derivative\ mass\ (g)}{Initial\ EPS\ volume\ (g} x100$$

Spectrometry at the Academy of Science and Technology. Perform spectrophotometry using an FT-IR meter.

2.2.6. Method to determine the monosaccharide composition of EPS

The GC-MS method was used to determine the hydrolyzed monosaccharide composition from ESP, which was chemically converted to a volatile structure by the following processes: hydrolysis, reduction and acetylation . These structures were analyzed in the GC-MS system.

2.2.7. Method for determining and predicting the structure - linkage of EPS

EPS is converted to volatile methylated alditol acetates (PMAAs). This process also allows the determination of the binding monosaccharide composition in the EPS (Refer to Wang et al. 2019's procedure) . Finally these structures were analyzed in the GC-MS system.

2.2.8. Method for determining the overall structure of EPS

The experiment was carried out at the Laboratory of Analytical Chemistry, University of Science, VNU, Ho Chi Minh City. HCM by NMR method.

2.2.9. Method to determine the biological activity of EPS

2.2.9.1. Investigation of the antioxidant activity of EPS by free radical scavenging ABTS $^{+}$

The EPS sample was reacted with ABTS solution in PBS pH 7.4 buffer, for 30 minutes, then was measured with OD 734nm and evaluate the free radical scavenging ability.

2.2.9.2. Methods for determination of mitotic resistance

The SRB (Sulforhodamin B assay) toxicity test was performed according to the process of Nguyen, TMN et al. (2017). The experiment was carried out at the Genetics Laboratory – SHPT, University of Natural Sciences, Ho Chi Minh City. HCM.

2.2.9.3. Test method for tyrosinase inhibitor activity

The experiment was carried out at the Biochemical Laboratory, University of Science, VNU, Ho Chi Minh City. HCM .

2.2.10. Data processing methods

Data are represented by mean ± standard deviation (mean ± SD). Microsoft Excel 2013 software is used for calculating figures and plotting graphs. The experiments are repeated at least 3 times. A p-value < 0.05 is considered statistically significant.

CHAPTER 3 . RESULTS AND ARGUMENT

3.1. Survey of liquid-static culture conditions for *C. sinensis*

3.1.1. Effect of nitrogen source on biomass and EPS complex

This study investigates the effect of different nitrogen sources such as peptone, yeast extract and silkworm pupae on biomass production, EPS biosyndissertation and ABTS+ free radical scavenging capacity of EPS [by] C. *sinensis* liquid-static cultures at different times of 10, 20, 30 and 40 days.

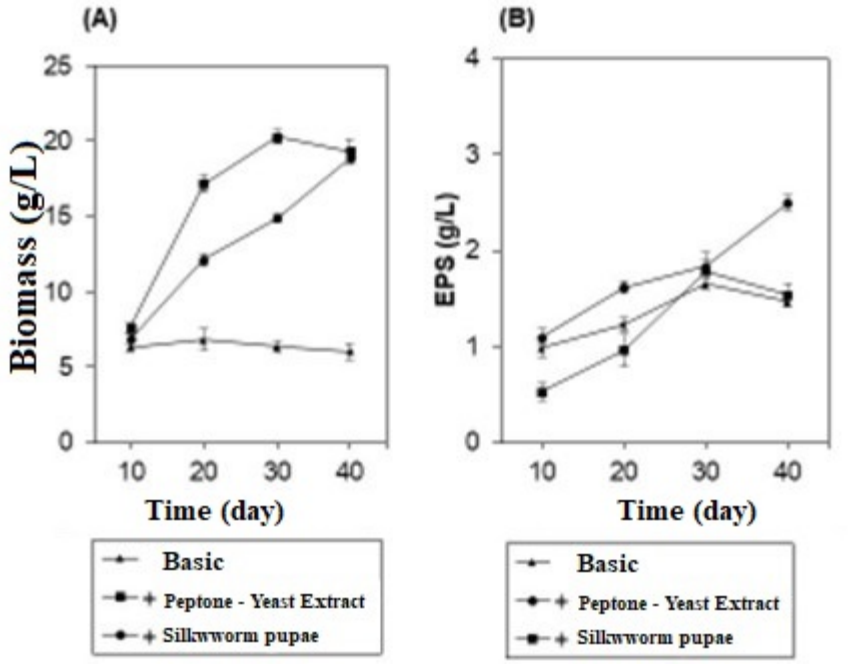

Figure 3.1. Effect of nitrogen sources on biomass generation (A) and EPS complexes (B) over time by liquid-stationary culture C. sinensis

In summary, suitable nitrogen sources in liquid-static culture for *C. sinensis* and EPS complexes are peptone and yeast extract. The highest collection time of EPS complex was at day 40 (2.5g complex EPS/L) corresponding to biomass yield of 19.3g SKK/L. Dong Yao et al. (2005) also suggested that peptone and yeast extract are important and suitable nitrogen sources in *C. sinensis culture* [76].

3.1.2. Effect of nitrogen source on EPS composition in EPS complex

The survey results showed that the nitrogen source plays a very important role in the EPS complex biosyndissertation of *C. sinesis* fungi . The simultaneous presence of polysaccharides and proteins in the EPS complex may be related to the existence of bioactive polysaccharide-protein complex segments. This is an interesting suggestion for further studies related to the examination of EPS segments after protein separation.

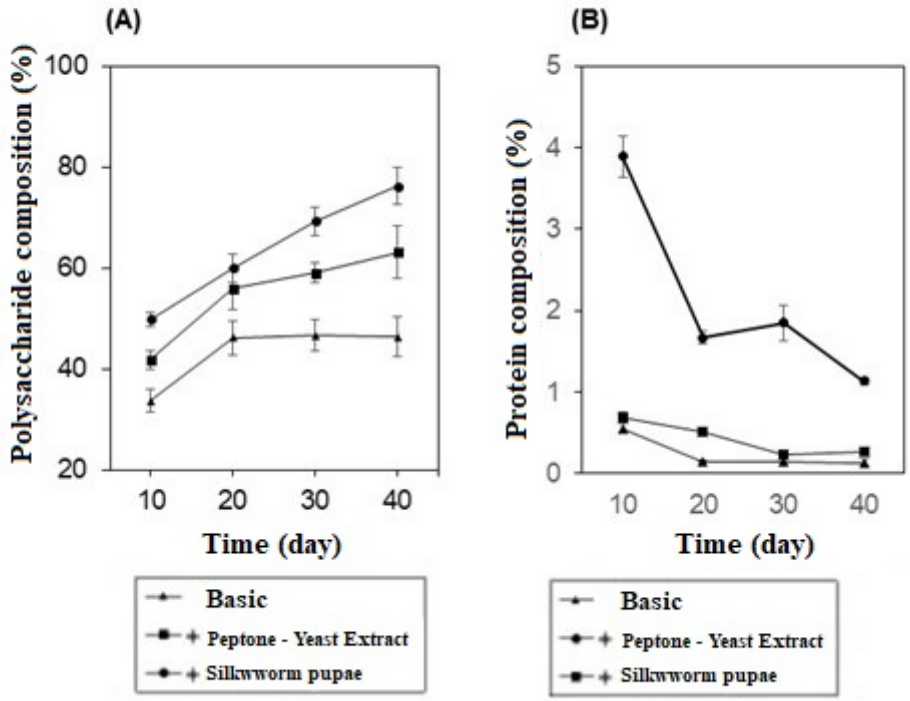

Figure 3.2. The replace change about wall polysaccharide (A) and protein (B) segment in separated EPS extract from 3 lips school other together according to time time feed transplant from 10 to 40 days

3.1.3. Effect of nitrogen source on the in vitro antioxidant activity of EPS . complex

The survey results show that the ABTS+ free radical arresting activity of the EPS complex varies significantly depending on the nitrogen source used in the liquid-static culture of *C. sinensis*. The EPS complex obtained at day 40 from a peptide supplementation medium and high in yeast at a survey concentration of 0-6 mg/mL exhibited the highest ABTS+ free radical capture activity, followed by the EPS complex from silkworm pupa supplementation medium. The lowest is the EPS complex from the basal environment (Figure 3.3A).

Changing different nitrogen sources in the culture medium can alter the polysaccharide composition and bioactivity of the EPS complex. In this study, peptone and yeast extract were suitable nitrogen sources to be added to the liquid-static culture of *C. sinensis* to produce EPS with free radical scavenging activity.

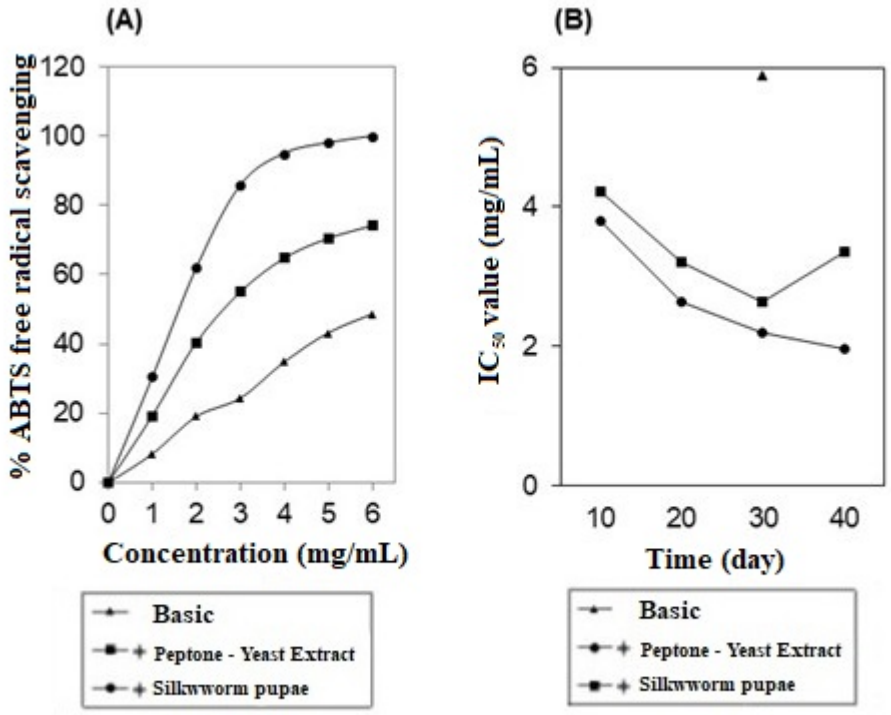

Figure 3.3. (A) ABTS+ free radical binding activity of EPS complexes obtained from different culture medium at day 40. (B) The change in ABTS+ free radical binding capacity of EPS complexes over culture time is expressed in IC_{50} (mg/mL).

In addition to the basic liquid-static culture medium of *C. sinensis* fungi, according to the research results of some authors, vegetable oils added to the liquid-static culture medium of *C. sinensis* will stimulate the biosyndissertation and bioactivity of EPS. Therefore, the next task was to investigate the liquid-static culture of *C. sinensis* fungi that stimulates the biosyndissertation and bioactivity of EPS with vegetable oil.

3.2. Survey on liquid-static culture of *C. sinensis* Stimulating the biosyndissertation and bioactivity of EPS by vegetable oil

3.2.1. Changes in biomass and EPS of C. sinensis in culture medium supplemented with vegetable oil

According to the reference, the authors added some vegetable oil to the liquid culture medium *C. sinensis* . In this study, coconut oil and sunflower oil were added in the range of 1-5%. Olive oil alone gives the maximum results at 5%. Therefore, conducting a survey to add olive oil in the range of 1-10%.

After 40 days of culture by static liquid method, biomass and EPS were obtained from *C. sinensis* fungi culture with coconut oil concentration from 1 – 5% and sunflower oil from 1 – 5%, olive oil 1 – 10%.

3.2.1.1 Survey results on the environment supplemented with coconut oil

In cultures supplemented with coconut oil, concentrations of 1-5% of *C. sinensis* growth and EPS generation had statistically significant differences in EPS content between check sample batches and experimental batches. The results showed that coconut oil supplementation at concentrations of 1-5% increased EPS syndissertation in fungi. Specifically, the dry EPS content reached the highest 2.54 ± 0.13 g/L in D4, the lowest 1.60 ± 0.01 in lot D1. Thus, the addition of coconut oil to the culture medium stimulated an increase in EPS syndissertation of *C. sinensis* fungus from 1.5 g/L to 2.54 g/L. A 1.66-fold increase compared to the population in batch D4 added 4% coconut oil to the culture. This increase is consistent with the increase in fungal biomass (Figure 3.4).

3.2.1.2 Survey results on the medium supplemented with sunflower oil

Culture medium for *C. sinensis* supplemented with sunflower oil concentration from 1 to 5% also increased the dry biomass yield and EPS. In the medium supplemented with sunflower oil, the amount of EPS obtained gradually increased when the oil concentration increased in the range of 1-3%. After 3% oil concentration, the amount of EPS formed started to decrease. The highest EPS collected in the medium supplemented with 3% sunflower oil was 2.56 ± 0.07 g/L, an increase of more than 1.5 times compared with the control (1.52 ± 0.22) ($p>0.05$).). The results obtained are similar to the results of Hsieh et al. in the survey of adding sunflower oil to the culture medium of *G. frondosa* [80].

3.2.1.3 Survey results on the environment supplemented with olive oil

The supplement of olive oil from 1 to 10% to *C. sinensis* culture medium stimulated EPS biosyndissertation significantly increased compared to the EPS medium (Figure 3.6). The volume of EPS increased sharply by 2 to 4 times compared to controls. In which, the highest was in the 5% olive oil supplementation medium with 5.3 ± 0.38 g / L, a 4-fold increase compared to the corresponding environment of 1.54 ± 0.03 g / L ($p<0.05$). This survey result is consistent with the findings of Hsieh et al. that olive oil has the ability to stimulate the fungus *Grifola frondosa* to increase EPS production [69].

Beside biomass generation yield and protein-EPS complex mass are obtained. The research purpose of the dissertation is to understand biological activity in general, including ABTS+ free radical binding activity of the protein-EPS complex. Therefore, the next investigation involved analysis of polysaccharide content in the complex, ABTS+ free radical binding activity of the protein-EPS complex obtained from the added medium of coconut oil, sunflower oil and olive oil.

3.2.2 Investigation of ABTS+ free radical arresting activity of protein-EPS complex precipitation obtained from three oil-supplemented medium

The ABTS+ capture activity of EPS obtained from an environment with an olive concentration of 2% or more was higher than that of EPS in the control medium (IC50 = 3067.48

µg/ml). The highest was a 5% olive medium with an IC50 = 1713.02 µg/ml, nearly 2 times higher than the control. Research by Yang et al. (2005) [65], Sharma et al. (2015) [5] showed that EPS obtained from *C. sinensis* has antioxidant, immune system modulation, anti-tumor, anti-aging activities, etc. Research by Yan et al. (2014) also showed that EPS segments obtained from fungal biomass and culture fluid has a high ability to capture hydroxyl radicals and ABTS+ [79].

In general, the ABTS+ free radical ability of EPS complex obtained from environmental supplementation of sunflower oil and coconut oil is not different from that of check sample and is not as good as olive oil supplementation. Therefore, olive oil supplementation medium was selected for further experiments involving appropriate time surveys for biomass and EPS capture in olive oil-supplemented cultures.

3.2.3 Investigate the appropriate time to collect biomass and EPS in culture fluid supplemented with olive oil

In this study, samples were taken at 10, 20, 30, 40, 50 and 60 days form *C. sinensis* culture liquid-static culture medium supplemented with olive *oil* to obtain biomass and EPS precipitate.

The process of development of the fungus increases with the culture period. The evidence is that the content of fungal biomass increases from 0 days to 40 days of culture. Specifically, the growth increased the most from 10 days to 20 days (up 5.44 times), to 30 days, the fungal biomass increased by 1.55 times compared to 20 days and increased by 1.22 times when collected at 40 days but this process did not increase much after 40 days of culture.

However, according to the general assessment, adding 5% olive oil will stimulate the fungi growth to thrive, biomass reaches 29.06± 1.06 g/L, while EPS syndissertation is also 5.3 ± 0.38 g/L compared to 1.52 ± 0.22 g/L) and better than coconut oil and sunflower oil supplements. Therefore, a 5% olive oil-fortified culture medium was selected for follow-up studies related to optimization of EPS syndissertation in olive oil supplementation medium.

3.3 Optimization of EPS syndissertation in olive oil supplemented medium

From the obtained results, it can be predicted that the optimal medium for liquid-static culture of *C. sinensis* has the following composition : potato extract (200 g/L), sucrose (48.69 g/L) , yeast extract (4 g/L), peptone (6.77 g/L), olive oil (5.27 %), KH_2PO_4 (0.5 g/L), K_2HPO_4 (0) ,5 g/L), $CaCl_2$ (0.5 g/L), $MgSO_4$ (0.2 g/L), pH 6-7. Experimental culture test on the optimal predicted medium obtained the results shown in Table 3.8.

Table 3.8. Cultivation results of *C. sinensis* on optimal predicted medium

Sample	IC_{50} (µg/mL)	EPS content (g/L)	Polysaccharide content (%)	Protein content (%)

Check Sample	3067.48	1.71	31.09 ± 0.98	1.79 ± 0.57
Predicted results	2304.34	5.75	53.01 ± 0.00	5.02 ± 0.01
Experimental results	2216.76	5.28	51.67 ± 3.82	4.45 ± 0.27

The results showed that there was no significant difference ($p<0.05$) between the experimental results and the predicted results. And both were better than check sample, with regard to EPS content, polysaccharide component % and protein content. The IC_{50} values of EPS obtained from optimal predictive environment and experimental culture were both lower than check samples. Thus, the optimization results are consistent with experiment.

Since the object of the dissertation is EPS obtained from *C. sinensis* liquid and static culture, it is necessary to conduct a study to remove olive oil from the fungus fluid with organic solvents in order to increase the efficiency of EPS extraction as well as to help evaluate the effect of olive oil on the bioactivity of EPS formed in liquid-static fungal culture medium *C. sinensis*.

3.3 Developing a process for extracting EPS from olive oil supplemented medium

3.3.1 Oil type solvent determination results

Three solvents used for the investigation were petroleum ether (PE), hexane and diethyl ether (DE). The effectiveness of the oil-based solvent was evaluated based on the delamination time, the obtained polysaccharide content, the lipid content in the sample before and after de-oiling and the bioactivity of the EPS after de-oiling. PE and hexane solvents pull olive oil out of the fungal culture more quickly and efficiently. The DE solvent removed the oil from the fungal culture for a longer time.

3.3.2 Polysaccharide and lipid content survey in culture after de-oiling

The fungal fluid after being de-oiled was determined the polysaccharide and lipid content. The ability to remove olive oil from the liquid-static culture of *C. sinensis* decreased in the order of DE > PE > Hexane. However, the residual polysaccharide content in the sample has the opposite trend with DE < PE= hexane. Therefore, it is necessary to conduct antioxidant activity test of the samples after oil removal to assess how the solvent used as well as the lipid content in the sample affects the antioxidant activity of the solution obtained after oil removal. type of oil to decide on the appropriate oil type solvent.

3.3.3 Investigation of ABTS+ free radical scavenging activity of oil samples

Samples after being removed oil were examined with ABTS+ free radical capture. The results showed that the oil type samples all had a higher percentage of free radical arrest than the non-oil samples. The sample which was de-oiled by PE and DE solvents captured > 50% of free radicals. The PE solvent oil sample had a lipid content of 41.75 percent lower than the hexane sample

with 48.59 percent and free radical capture was 58.69 percent, almost twice as high as the sample de-oiled with hexane solvent (37.98 percent). The results showed that the larger the fluid cultured with the oil, the higher the ability to capture free radicals. Although DE solvents remove the most oil from the culture, due to the high loss of polysacharide content in the sample, the free radical capture activity is only equivalent to that of PE-type cultures. Therefore, choose PE solvent to type oil to conduct further experiments.

After remove oil, the next investigation involved obtaining the EPS-protein complex by precipitate with ethanol.

3.3.4 EPS-protein complex acquisition survey

The EPS-protein complex were obtained by dissovling the fungal culture after de-oiled and ethanol 96^{o} with ratio between the culture and ethanol is 1:3 ; 1:4 and 1:5

After obtaining the EPS-protein complex, the polysaccharide and protein content of the raw EPS-protein sample were determined. The results showed that at the precipitation ratio between fungal culture fluid and ethanol 960 1:3 (v/v) the mass of EPS-protein precipitate was lowest as 0.0154 g/mL. At a ratio of 1:4 it had highest as 0.0171 g/ml. At a ratio of 1:5, the resulting precipitate mass decreases to only 0.0166 g/mL. Therefore, a precipitate ratio of 1:4 was chosen for the extraction of EPS from the liquid and static culture of *C. sinensis* .

After determining the optimal conditions for the removal of olive oil from the *C. sinensis fungal culture* and the EPS-protein precipitation, the next step is to investigate the purification to remove the protein from the EPS-protein complex. obtained the EPS segments after protein removal and studied the modification by sulfate to improve the biological activity of the EPS segments.

3. 5. Acquisition, purification and sulphate modification improve the bioactivity of EPS segments

We first investigated the purification of EPS by removing proteins from the EPS-protein complex . In the framework of the dissertation research , Sevag, TCA and Alcalase methods were investigated to remove proteins to determine which treatment method is the most effective based on the free radical scavenging activity of EPS after protein removal.

3. 5.1.1 . Obtaining and purifying EPS

Table 3.1 1. Comparison of protein removal capacity of TCA 10%, Sevag 4:1 and Alcalase enzyme 20 UI/m L

Protein removal method in raw EPS	**Polysaccharide content (%)**	**Protein content (%)**	**% free radical scavenger**
TCA 10%	41.23 ± 1.54	2.56 ± 0.26	35.42 ± 3.24

Sevag 4:1	62.72 ± 0.18	1.65 ± 0.46	60.76 ± 2.18
Alcalase 20UI/ml	**69.2 ± 1.83**	**1.57 ± 0.22**	**68.98 ± 2.05**

Protein removal results from raw EPS by three methods TCA, Sevag and Alcalase are shown in Table 3. 1 1. In which, using Alcalase is the best method to remove protein from crude EPS-protein complex.

After significant removal of protein from the EPS-protein complex in the fluid, the EPS content increased significantly. Because EPS is the research object of the dissertation, the next task is to collect and investigate the biological activities of the EPS segments obtained by gel filtration and Tangential Flow Filtration.

3.5.2. Acquiring and investigating the biological activity of EPS segments

3.5.2.1 . Acquisition of EPS segments by gel filtration

Using Sephadex G-100 gel filter chromatography obtained 2 EPS segments: EPS I tube 35) and EPS II. In EPS I, the amount of protein is higher than polysaccharides. In contrast, in the EPS II segment, polysaccharide levels are higher than proteins, both of which are higher than EPS I. In both segments, the EPS and protein content curves share the same peak in the tubes located together.

3.5.2.2. Acquisition of gel-filtered EPS segment after protein removal with Alcalase 20 UI/ml

After using Alcalase 20 UI/mL to remove proteins from the EPS complex, the EPS segments were separated by Sephadex G-100 gel filtration chromatography.

Results after chromatography obtained 2 EPS segments. EPS I (25-31) and EPS II (61-76th tube). The OD 280 nm corresponds to a sharp decrease in the protein content at the two absorption peaks of both EPS I and EPS II. Similarly, the absorption peak of polysaccharide was also lower than before treatment. This proves that the polysaccharide and protein are bound together in the form of a protein-polysaccharide complex. Alcalase treatment has not hydrolyzed all the proteins in the complex.

3.5.2.4. Investigation of anti-mitotic activity of EPS I and EPS II segments

Investigation of anti-mitotic activity of EPS I and EPS II segments through cytotoxicity by SRB test on Hep-G2 liver cancer cell line.

The results obtained EPS II have higher cytotoxic activity compared to EPS I. The IC_{50} value of at the concentration of 32.38 ± 3.65 µg/ml of the EPS II segment can be considered as likely cytotoxic. However, the bioactivity of the EPS segments can be improved through modification of their chemical structure. One of the approaches is sulphation modification, adding sulfate groups to the EPS structure to increase their biological activity. Therefore, the next study is the sulphation modification of EPS.

Gel filtration to obtain EPS segments as shown above is a laboratory-level approach. However, in practical production at the pilot level, or moreover, at the level of industrial production, Tangential Flow Filtration is often used. Therefore, the next study was to collect the EPS segment from the liquid-static culture of *C. sinensis* by Tangential Flow Filtration.

3.5.3. Survey on acquisition and purification of EPS segments from C. sinensis liquid-static culture by Tangential Flow Filtration

In this experiment, EPS was cultured and precipitated with ethanol 96% at 1:4 ratio (v/v) for 24 hours at 4^{o}C, with an efficiency of 2.383g/L. Dissolve 10 g of raw EPS and proceed to collect EPS segments by Tangential Flow Filtration with 100kDa membranes, from 100 to 750kDa and >750kDa. The analysis results of total soluble polysaccharide showed that the polysaccharide content of the EPS segments was higher than that of the raw EPS sample. After removing protein by Alcalase, polysaccharide content increased in all EPS segments. The above results show that the studied EPS contains a large amount of polysaccharide with MW from 100-750 kDa. This proves that the Tangential Flow Filtration method is suitable for obtaining and purifying EPS sample segments.

Because the Hep-G2 cytotoxic IC_{50} value of the two segments EPS I and EPS II is at best 32.38 ± 3.65 μg/ml, the threshold of 30 μg/ml has not been exceeded. Therefore, the modification and upgrading of their biological activity is necessary. This was followed by a sulfation modification of EPS segments obtained after Tangential Flow Filtration in order to enhance their biological activity.

3. 5.4. Sulfation results of EPS segments after Tangential Flow Filtration

Perform sulphation reaction of three EPS segments obtained after Tangential Flow Filtration and remove protein by Alcalase from EPS-protein complex by CSA/Pyr method according to the ratio EPS/SO_3.Py is 1:5 and 1:7 (w/w) respectively in the Argon atmosphere at 90 ^{o}C, corresponding to the time of 1h, 2h and 3h. The results of sulfatedation of 3 EPS segments obtained from the EPS complex after Tangential Flow Filtration are shown in Table 3.14.

Table 3.14. Results of sulfatedation of three EPS segments obtained after Tangential Flow Filtration

Ratio EPS/SO_3.Py (w/w)	**Time (h)**	**<100 kDa**	**100-750 kDa**	**>750 kDa**
1:5	first	Not achieved	S=O (**at 1240 cm $^{-1}$**) **DS=2.32; 90.375%** at t=2h	S=O (1200 cm $^{-1}$)
1:5	2			
1:5	3			
1:7	first		S=O **(at 1208 cm $^{-1}$) DS=1.64; 55.348%** at t=2h	S=O (1200 cm $^{-1}$)
1:7	2			
1:7	3			

The 100-750kDa segment has a higher DS value than the other 2 segments, especially the highest DS at the ratio EPS/SO_3 .Py 1:5 (**DS=2.32; 90.375% at t=2h**) ; in which the segment < 100kDa does not occur sulfate reaction and the segment > 750 kDa gives a DS value < 1.

The purpose of the dissertation is to study how to obtain biologically active EPS. Therefore, the next step is to investigate the biological activity of the EPS segments after the sulfate upgrade.

3.5.4.1. Survey results of ABTS+ free radical arrest activity of three EPS segments after sulfation

Unsulphated EPS showed better antioxidant results in the small MW segments than in the larger MW EPS. The EPS segment <100kDa had the best antioxidant activity of 66.51% with IC_{50} 3026 µg/mL. The lower MW EPS segment had higher antioxidant activity than the higher MW EPS segment possibly due to its better degree of flexibility and solubility. This result is similar to the results published by Li et al (2021) who showed that polysaccharide molecules isolated from *Cordyceps* with lower MW had higher superoxide anion scavenging activity than those with MW which higher [84].

3.5.4. 2. Investigation of anti-Tyrosinase activity of three EPS segments after sulfation upgrade

The results recorded two experiments shown that tyrosinase resistance of S-EPS samples of 100-750 kDa at two ratios of 1:5 and 1:7, respectively 852.7 and 927.4 µg/mL. The remaining S-EPS samples were above 4000 µg/mL, with no Tyrosinase resistance. A 1:5 S-EPS sample gave a best tyrosinase resistance of 852.7 µg/mL. Tyrosinase inhibitory activity survey results showed that 100-750 kDa S-EPS samples had the best results with DS=2.32 efficiency. The 100-750 kDa S-EPS sample is the most obvious sulfate sample. On the FT-IR spectrum there are C-O-S oscillations of approximately 815 and S=O at 1240 cm-1. In addition, the ABTS+ free radical antioxidant activity of S-EPS 100-750 kDa was significantly improved, increasing from 60.94% of natural EPS to 98.62%, an increase of more than 38%.

3. 6. Determination of composition and chemical structure of EPS from *C. sinensis* culture fluid

3.6.1. Investigate Monosaccharide unit of EPS

In order to determine the correct monosaccharide composition unhindered by yeast and peptone, in this study in addition to the basic liquid culture-static fungus *C. sinensis* containing peptone and high yeast, the other cultures were being used without contain peptone and high yeast but added five other substrate sources as 4% glucose, fructose 4%, sucrose 4%, peptone 4% and mixtures thereof with 1% each.

The results showed that all tests (NT) contained three monosaccharide components: glucose, mannose and galactose in different ratios. Wang et al. (2009) also obtained polysaccharides from *C. sinensis* biomass composed of glucose, mannose and galactose at a ratio of 2.8:2.9:1, MW in the range of 8.1 kDa [87]. The results of the study showed that: The EPS-HM fraction with MW 6250 kDa contains four monoscaccharide components: mannose, glucose, galatose and ribose with a ratio respectively 1,71 : 0,94: 1: 0,09. While in EPS-LM segments had low MW in the range of 40 – 360 kDa contains only three monosaccharide components: mannose, glucose and galactose in a ratio respectively 7,76 : 13,87 : 1.

3.6.2. Determination of glycoside binding forms in EPS

After determining the monosaccharide composition of EPS from the culture of *C. sinensis*, then determine the glycoside linkages connecting monosaccharide in EPS. The results are summarized in Table 3.18.

Table 3.18. Glycoside linkages in EPS of 5 medium

Order	Linkages types	Ratio (%)					
		Glucose	Fructose	Sucrose	Peptone	Mix	E/C
1	→2)-α-D-Man*p*	1,31			16,98		
2	→3)-α-D-Glc*p*	11,48	0,63			0,36	14,01
3	→3)-α-D-Man*p*-(2→	8,98	15,99	19,28	5,27	17,67	12,83
4	**→3)-β-D-Man*p*-(1→**	**54,19**	**55,33**	**68,16**	**29,17**	**55,58**	**46,44**
5	→4)-α-D-Man*p*	0,20					
6	→4)-α-D-Man*p*-(3→	0,65					

7	→6)-α-D-Man*p*	0,29					19,84
8	→6)-α-D-Man*p*-(2→	2,98					
9	→6)-α-D-Man*p*-(3→	0,92					
10	α-D-Man*p*-(2,3,6→	1,71	5,71	4,74	6,26	7,19	
11	α-D-Gal*p*-(2,3,4→			0,28			
12	α-D-Man*p*-(2,3,4,6→		1,20			0,71	0,65
13	→4)-α-D-Glc*p*-(2→			0,77			

EPS obtained from all *C. sinensis* culture medium tests had a primary circuit of →3)-β-D-Manp-(1→, of which in 4 medium glucose, fructose, sucrose, mix and sample were all high, respectively 54.19%; 55.33%; 68.16%; 55.58% and 46.44%. While the ratio of branch circuits in different trials is different. Two branch circuits appeared in all 5 experiments: →3)-α-D-Manp-(2→ and α-D-Manp-(2,3,6→ at different rates. EPS obtained in the glucose-containing medium test has the most branch vessels present. In medium contained sucrose and peptone, there was no branch chain →3)-α-D-Glcp. Particularly in the environmental test of peptide supplementation, branch chains →2)-α-D-Manp (16.98%) were present. This demonstrates that in addition to the peptone environment, *C. sinensis* produces very little EPS. The association of EPS in the peptone supplementation test was also very different from the rest of the trials.

Research results of other studies show that monosaccharide composition of EPS is also glucose, mannose and galactose in different proportions. In which, incomplete glucose accounts for the highest percentage. These EPSs all have the main circuit →3)-D-Glc *p* -(1→, while the main circuit in 5 independent variables in this study is →3)-β-D-Man *p* -(1→ . *This is the new result recorded in the EPS structure obtained in the liquid-still culture of C. sinensis fungus provided by Dr. Truong Binh Nguyen. In addition, the presence of multiple branch chains in the EPS structure in this study also showed marked differences.* While EPS obtained in other studies found only 1 to 2 branch circuits, EPS obtained in all 5 independent variable in this study had more than 2 branch circuits. This says that the structure of EPS varies depending on each individual fungi as well as the medium in which they are cultured..

3.6.3. Surveying the structure of EPS by NMR

The configuration of the glycosidic bond in the polysaccharide was analyzed by ^{1}H NMR. Compared with other spectrograms of natural products, the signals of polysaccharides collected in a narrow range δ 4.3 – δ 5.9 ppm corresponding to the signals of 3 sugars. EPS obtained from all 5 independent variable above had peak signal at δ 5.16 ppm and in the range δ 4.2 – δ 4.3 ppm. The results showed a strong signal in the range δ 5.03 ppm which is the signal of ^{1} H anomeric of α-D-mannose and β-D-galactose.

All tests have a primary circuit of →3)-β-D-Manp-(1→, Four medium: glucose, fructose, sucrose and mix shown high percentage. ^{1}H NMR spectrum gives signals at δ 5.16 ppm, δ 4.2 – 4.3 ppm.

3.7. Discuss the research results of the dissertation and propose the process of liquid-static culture of *C. sinensis fungus* to generate EPS with biological activity

This dissertation focuses on studying the static liquid culture of *C. sinensis* as the basis for optimizing the technological process of producing this medicinal fungi in Vietnam. Study on obtaining EPS and bioactive EPS segments from fungal culture fluid and studying their bioactivity enhancement. Some of the following issues have been clarified within the framework of the dissertation.

The first discussion is related to the screening study of the liquid-static culture of *C. sinensis* . In the review, the advantage of liquid culture of *C. sinensis* compared with semi-solid culture was mentioned . However, most of the studies on liquid culture of *C. sinensis* so far have mainly focused on liquid culture studies combined with stirring at speeds in the range of 100-150 v/min. Not much mention has been made of the liquid-static culture *of C. sinensis* . Regarding the composition of *C. sinenesis* fungal culture medium, they are basically quite similar. Sources of Cacbon are usually came from sucrose and glucose or cornstarch. The source of Nitrogen is yeast extract and peptone and some inorganic salts that provide trace minerals. The pH of the culture medium is usually acidic in the range of 4.0-4.5. There are also studies conducted at pH 7.0 (Wu, Chen and Hao. 2009).

Biomass generation yields and EPS are also different. The highest was in the study of Cha et al. (2007) which achieved corresponding yields of biomass and EPS of 54.0 g/L and 28.4 g/L. The study of Wu, Chen and Hao 2009 liquid cultures of *C. sinensis* stirred 150 v/min, pH 7.0 gave a relatively low biomass yield of 12.3 g/L, but the yield of EPS production is quite high up to 24.5 g/L. This proves that artificial liquid culture of *C. sinensis* still needs to be studied further related to the screening of culture medium composition, liquid-stirring, liquid-static or semi-solid culture.

The research results of the dissertation showed that the biomass production of *C. sinensis* in liquid-static culture was lowest on the basic medium without adding nitrogen. The highest biomass collection time was on day 20 with 6.8 g of dry biomass (SKK)/L. Before the fungus enters

equilibrium and declines (Figure 3.1A). Meanwhile, on medium supplemented with peptone and yeast extract , the peak time of fungal biomass of 20.3 g SKK/L on the 30th day of culture was about three times higher than the biomass generated on the basic medium. (6.8 g SKK/L). The biomass of *C. sinensis* produced on the medium supplemented with silkworm pupae extract was also better than on the basal medium. At day 30, the fungal biomass on the medium supplemented with silkworm pupae extract reached 14.9 g SKK/L which was significantly higher than the basal medium (6.4 g SKK/L), but lower than that of the basal medium. 20.3 g SKK/L on medium supplemented with peptone and yeast extract. It is interesting that on the medium of silkworm pupae extract, after the 30th day, the fungal biomass continued to be generated until the 40th day reached 18.9 g SKK/L.

The second discussion is related to the addition of olive oil to the liquid-static culture of *C. sinensis* stimulate biomass and EPS.

The study results also showed that coconut oil is not suitable for the growth and synthesis of EPS of liquid and stationary cultured *C. sinensis* fungi because the main component of coconut oil is saturated fatty acid, containing only a small amount of 6.2% unsaturated fatty acid oleic acid. Therefore, coconut oil is not suitable for fungi growth [78]. While sunflower oil and olive oil are high in unsaturated fatty acids, they are more suitable for addition to liquid-static cultures that stimulate *C. sinensis* growth and synthesis of EPS. The results of this survey are similar to the study of Hsieh et al. (2008) when adding 0.1-1% sunflower oil and olive oil to *G. frondosa fungus culture medium.* [80] . This is reasonable because sunflower oil and olive oil have a high content of oleic acid unsaturated fatty acid from 55-75%, which helps fungi to easily grow to increase biomass and synthesize EPS [61] . The survey results are quite interesting. The addition of sunflower oil resulted in a higher yield of fungal biomass compared with the addition of olive oil. In contrast, olive oil supplementation resulted in higher EPS content than sunflower oil supplementation.

However, according to the general assessment, adding 5% olive oil will stimulate the fungus to thrive, the biomass will reach 29.06± 1.06 g/L, and the total EPS will also be high 5.3 ± 0.38 g/L. L vs 1.52 ± 0.22 g/L) and better than coconut oil and sunflower oil supplementation. Therefore, the culture medium supplemented with 5% olive oil was selected for further studies related to the optimization of EPS syndissertation in the medium supplemented with olive oil.

The third discussion was related to the optimization of the liquid-static culture medium composition of *C. sinensis* with the addition of olive oil to stimulate biomass generation and EPS.

In the research of this dissertation, the liquid-static cultures aimed at obtaining EPS were performed by using three factors at 3 levels of 15 formulations including three repetitions of the central points. Table 3.4 shows experimental results compared to predictive model results using Minitab 17 software. In general, the results obtained from experiments and models do not have significant differences.

C. sinensis liquid-static culture medium supplemented with olive oil showed the ability to attract oil and activity of the test sample after oil removal with PE and DE solvents were better than hexane solvent. DE solvent's ability to draw oil out of the solution is the best. However, a large amount of polysaccharide is lost. The free radical scavenging activity of the PE and DE solvent samples was almost the same. Therefore, PE solvent was selected to remove the oil for further experiments.

The fourth discussion concerns the extraction of the EPS-protein complex from the liquid-static culture of *C. sinensis* with olive oil, after the oil has been removed.

The results showed that, at the ratio of precipitation between fungal culture solution and ethanol 96^0 1:3 (v/v), the volume of EPS-protein precipitate was the lowest of 0.0154g/mL. At 1:4 ratio is the highest 0.0171 g/ml. At the ratio 1:5, the volume of precipitate obtained was reduced to only 0.0166 g/mL. Therefore, a 1:4 precipitation ratio was selected for the extraction of EPS from the liquid-static culture of *C. sinensis* . The results of this study are similar to those of Kim and Yun (2005) on *C. militaris* and *C. sinensis* ; Angelis and Sandra (2009) on Macromycetes; Tran Minh Trang (2013) on *C. sinensis*. Ratio 1:4 (v/v) between liquid-static culture and 96^0 ethanol to obtain EPS-protein complexes from *C. sinensis* cultures supplemented with olive oil was optimal, and was selected for further studies.

The fifth discussion is the purification of EPS segments from the precipitated EPS-protein complex which were obtained from the *C. sinensis* fungal culture supplemented with olive oil.

Results of protein removal from raw EPS by three methods TCA, Sevag and Alcalase are shown in Table 3.11. In particular, using alcalase is the best method to remove protein from the EPS-crude protein complex. Specifically, the ratio of protein types differs between the three survey methods. The method using Alcalase had the highest protein removal capacity, reduced to only 1.57% with the highest obtained polysaccharide content (69.2%) ($p < 0.05$), with the highest free radical binding activity of EPS 68.98% higher than the other 2 methods. The TCA method caused a greater loss of polysaccharide and reduced free radical scavenging activity compared with the sample. The Sevag method also gave relatively high protein removal results, consistent with the results of Huang et al. (2009) using the Sevage method to remove proteins in mannan oligosaccharides, with a protein removal rate of 89.8% with 12 .2% mannan oligosaccharide loss [50] . However, compared to the method using Alcalase, it is still inferior. Therefore, Alcalase was chosen to be the method for removing protein from the EPS complex.

The sixth discussion concerns the investigation of the free radical scavenging activity of purified EPS segments.

Table 3.12 results show that after removing protein from EPS complex. By gel filtration chromatography G-100, two EPS segments I and II were obtained . The results show that EPS II has

higher cytotoxic activity than EPS I. Thus, the IC_{50} value at the concentration of 32.38 ± 3.65 µg/ml of the EPS II segment can be considered as potentially cytotoxic. However, the bioactivity of the EPS segments can be improved by modifying their chemical structure. One of the approaches is sulphation modification, adding sulfate groups to the EPS structure to increase their biological activity. Therefore, the next study is the sulphation modification of EPS.

The seventh discussion relates to the modification and upscaling of bioactivity of EPS fractions that have been purified by sulfation. Because in the investigation, the Hep-G2 cytotoxic IC_{50} value of 2 segments EPS 1 and EPS 2 has reached 32.38 ± 3.65 µg/ml, not exceeding the required threshold of 30 µg/ml. Therefore, the modification and upgrading of the biological activity of these two segments is necessary.

The results of sulfated segments showed that the DS values in the segments were significantly different (Section 3.3.4). Specifically, the 100-750 kDa segment has a higher DS value than the other two segments, at the ratio EPS/SO_3 .Py 1:5 (DS=2.32; 90.375% at t=2h) . While the segment < 100 kDa has no sulfate reaction occurs. The segment > 750 kDa gives a DS value < 1. From the above data, it can be concluded that the sulfate reaction conditions are suitable for 3 EPS segments from the EPS precipitate obtained from the liquid-static culture medium fungus *C. sinensis* is the ratio EPS/SO_3.Py (1:5 and 1:7) in 2h, at a temperature of 90^0 C. This is also a new highlight of the dissertation that successfully created sulfate derivatives from EPS which was obtained in a liquid-static culture medium of *C. sinensis.*

Tyrosinase inhibitory activity survey results showed that 100-750 kDa S-EPS samples had the best results with DS=2.32 efficiency. The 100-750 kDa S-EPS sample is the most obvious sulfate sample. On the FT-IR spectrum there are COS oscillations of approximately 815 and S=O at 1240 cm-1. Along with that, the ABTS+ free radical antioxidant activity of S-EPS 100-750 kDa was significantly improved, increasing from 60.94% of natural EPS to 98.62%, an increase of more than 38%.

In summary, sulfated modification changes the physicochemical properties of EPS and improves their biological activity. The sulfate group SO_3^{2-} significantly affects the bioactivity of the sulfated derivatives of EPS. In the research of this dissertation that successfully synthesized sulfated derivatives S-EPS under optimal conditions with the ratio EPS/SO_3 .Py = 1:5, 90 ^{o}C and 2h, reaching DS = 2.32 with efficiency 90.375%. The DS value obtained in this study is higher than in previous studies, which is very promising. This is a new point that opens the application direction of using EPS/SO_3 .Py method to improve sulphationization and improve biological activity of EPS obtained from liquid-static fungal culture *C. sinensis.*

The eighth discussion is the composition and chemical structure of EPS. Research results of other studies show that monosaccharide composition of EPS is also glucose, mannose and galactose

in different proportions. In which, Glucose isn't always seem to be the highest percentage. These EPSs all have the main circuit →3)-D-Glcp-(1→. Mean while the main circuit in 5 medium in this study is →3)-β-D-Manp-(1→. New results were recorded in the EPS structure obtained in the liquid-static culture of fungi *C. sinensis* was provided by Dr. Truong Binh Nguyen. Furthermore, the appearance of many branched circuits in the EPS structure in this study also shows a clear difference. While EPS obtained in other studies in Table 3.22 only shows the presence of 1 or 2 branch vessels. While EPS obtained in all five medium in this study have more than 2 branch vessels. This means that the EPS structure varies depending on the individual fungi as well as the environment in which they are grown.

General conclusions: Liquid-static culture *of C. sinensis* on different substrate sources gave different biomass and EPS. Monosaccharide composition of EPS in all treatments contained glucose, mannose and galactose in different proportions. In which, EPS of glucose and sucrose medium had the highest mannose content at 65.10% and 47.55%. EPS of the remaining 3 medium had the highest glucose content.

All medium have a main circuit of →3)-β-D-Manp-(1→ , in which 4 medium: glucose, fructose, sucrose and mix have a high ratio. ^{1}H NMR spectrum gives signal at δ 5.16 ppm, δ 4.2 – 4.3 ppm.

The structure of EPS has less branched with peptone as the substrate source. While the substrate source is glucose, EPS has many branched chains. EPS has a high molecular weight and a high galactose composition at the sucrose substrate source.

The research results of the four main contents of the dissertation allow to propose a process of liquid-static culture of *C. sinensis* in order to obtain biologically active EPS.

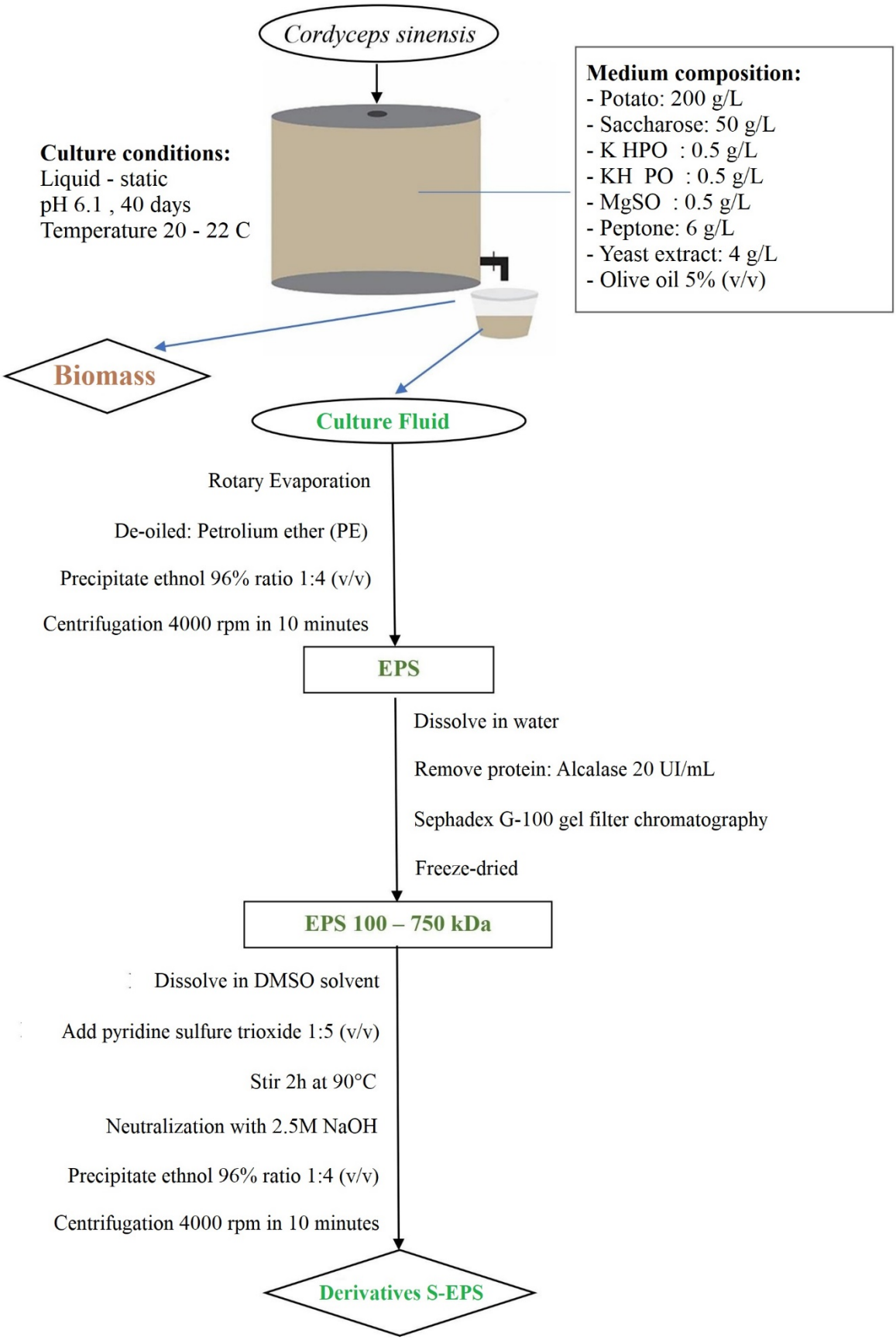

Figure 3.4. Proposing a process for culturing the biologically active C. sinensis fungi to produce EPS

CONCLUSIONS AND RECOMMENDATIONS

❖ Conclusions:

The research results of the dissertation allow the following conclusions:

- Peptone and yeast extract are the suitable nitrogen sources in liquid-static culture of *C. sinensis* to obtain EPS with better antioxidant activity than silkworm pupae.
- It was determined that the addition of 5% olive oil to the basal culture medium stimulated increased EPS syndissertation by the liquid-static cultured fungus *C. sinensis* .
- Purification of EPS was obtained from the liquid-static culture of *C. sinensis* by protein removal using Alcalase. Acquire the EPS segment 1 and EPS 2 by gel filtration chromatography and obtained the 100-750kDA EPS segment by tangential flow filtration .
- Upgrading the activity of the 100-750 kDa EPS segment with sulfation significantly improved in vitro antioxidant activity and tyrosinase inhibitory activity, which is the new contribution of the dissertation.
- Three main monosaccharide units have been identified in the EPS chain obtained from the liquid-static culture of *C. sinensis* , name as glucose, mannose, galactose, with the main chain 1,3-β-D-Manp, 2 branched vessels 2,3)-α-D-Manp and α-D-Manp-(2,3,6 in EPS structure. Substituting different carbon sources in culture may affect branch-chain structure, but does not alter the main-chain structure of EPS produced by *C. sinensis* .
- A technological process for liquid-static culture *of C.sinensis* has been proposed to generate fungal biomass and high EPS with biological activity from liquid-static culture of *C. sinensis* . This is the first study to develop a process for obtaining and purifying EPS from *C. sinensis* culture medium .

❖ Recomendations:

For the purpose of further completing the research contents in order to obtain the EPS active ingredient from the liquid-still culture of *C. sinensis,* the dissertation has some recommendations below:

- Continous research to create a liquid-static culture of *C. sinensis* in order to obtain higher yield of fungal biomass and EPS from the culture solution.
- Completing the process for obtaining EPS segments from culture fluid and generating bioactive sulfate derivatives at pilot scale, as a premise for the production of bioactive EPS derivatives.

NEW CONTRIBUTIONS FROM THE DISSERTATION

1. For the first time, a process for obtaining EPS from liquid and static cultures of *Cordyceps sinensis* fungi in Vietnam helps to actively create bioactive EPS sources.
2. The medium composition and optimal culture conditions have been determined for *Cordyceps sinensis* containing peptone and yeast extract in combination with 5% olive oil supplementation with 40 days of culture time to stimulate the growth and syndissertation of EPS.
3. Three major components of EPS have been identified from the liquid-static culture of *Cordyceps sinensis* as glucose, mannose and galactose in different proportions in the EPS segments.
4. Sulfate-EPS derivation was created by EPS/SO3.Py method with the ratio EPS/SO3.Py = 1:5, 90 oC and 2h, reaching DS = 2.32 with the efficiency of 90.375% higher than other methods in other previously published research. The EPS segments obtained from *Cordyceps sinensis* liquid-static cultures after being sulfated modified all showed significant bioactivity improvements. Especially the 100-750 kDa S-EPS segment.
5. The dissertation contributes to clarify the EPS structure obtained from the liquid-static culture of *Cordyceps sinensis* whose main vessel is →3)-β-D-Manp-(1→. The 1H NMR spectrum indicates the location of the anomeric Cs of Manp, Galp and Glcp..

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